

5           **Serine Protease and Topical Retinoid Compositions Useful**  
              **for Treatment of Acne Vulgaris and Production of Anti-**  
                              **Aging Effects**

Cross-Reference to Related Application

10           This Application claims the benefit of United  
States Provisional Application Number 60/037,605 filed  
on 12 February 1997, which is incorporated herein by  
reference in its entirety.

15           Field of the Invention

              This invention is related to methods for treating  
Acne Vulgaris and/or for producing anti-aging effects on  
the skin of a mammal, and compositions effective for the  
same. More specifically, the present invention is  
20           directed to the use of serine proteases, either alone or  
in combination with a retinoid compound in a  
pharmaceutical or cosmetic composition.

Background of the Invention

25           Acne Vulgaris is a disorder of the pilosebaceous  
unit that affects nearly all adolescents to some degree,  
as well as many adults. The initial lesion of the  
disease is believed to be due to hypercornification and  
hyperkeratinization of the infundibulum, a process that  
30           helps to transform the sebaceous follicle into a  
comedone. This disorganization of the epithelium may  
give rise to inflammatory lesions, as the infundibulum  
ruptures and sebum is introduced into the dermis.

5           Accordingly, traditional therapies are directed  
against the three major pathological processes which  
contribute to the development of Acne Vulgaris.  
Treatments such as topical retinoids work against the  
obstruction of the sebaceous follicle resulting from  
10 abnormal desquamation of the follicular epithelium.  
Hormonal agents target the androgen-stimulated increase  
in the production of sebum. Finally, antibiotics  
function to reduce and/or halt the proliferation of  
propionibacteria within the follicle which contribute to  
15 inflammation. Benzoyl peroxide, salicylic acid, and  
various cleansing agents are also employed for similar  
purposes. Topical retinoids are considered to be one of  
the most effective classes of comedolytic agents for the  
treatment of Acne Vulgaris, however their clinical  
20 efficacy is limited by their irritant effects.

Topical retinoids have also been used to produce  
anti-aging effects on the surface of mammalian skin.  
While they are known in the art as one of the most  
effective topical treatments available, these compounds  
25 are limited by their irritant effects.

It would be desirable to provide a method for  
treating Acne Vulgaris which is as effective as  
traditional acne therapies, but which is not associated  
with high levels of irritancy.

30           It would also be desirable to provide a method for  
producing anti-aging effects on the surface of mammalian  
skin which is as effective as retinoid treatments, but  
does not have the same irritant effects.

5        Summary of the Invention

          In accordance with the present invention, we have found a method for treating Acne Vulgaris and/or for producing anti-aging effects on the skin of a mammal comprising, consisting essentially of, or consisting of  
10        topically applying to the skin of a mammal an effective amount of a first topically active agent comprising a protease.

          In another embodiment of the present invention, we have found a method for treating Acne Vulgaris and/or  
15        for producing anti-aging effects on the skin of a mammal comprising, consisting essentially of, or consisting of topically applying to the skin of a mammal an effective amount of a first topically active agent comprising a  
20        protease in combination with a second topically active agent comprising a retinoid.

          In yet another embodiment of the present invention, we have found a pharmaceutical or cosmetic composition comprising, consisting essentially of, or consisting of:

- 25        a) a first topically active agent comprising a protease; and  
          b) a second topically active agent comprising a retinoid.

          The compositions and methods of this invention provide a unique, convenient means for treating Acne  
30        Vulgaris and/or for producing anti-aging effects on the skin of a mammal.

Brief Description of the Drawings

5           The file of this patent contains several drawings executed in color. Copies of this patent with said color drawing will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

10           The invention will be more fully understood and further advantages will become apparent when reference is made to the following detailed description of the invention and the accompanying drawings in which:

15           FIG. 1(a) is a representation which illustrates a cross-sectional view of the skin of a Rhino mouse one hour after treatment with fluorescently labeled trypsin. FIG. 1(b) is a representation which illustrates a cross-sectional view of the skin of a Rhino mouse four hours after treatment with fluorescently labeled trypsin. FIG  
20 (c) is a representation which illustrates a cross-sectional view of the skin of a Rhino mouse four hours after treatment with fluorescently labeled trypsin, following 5 days of daily treatment with 1% (w/v) trypsin.

25           FIGS. 2(a) and 2(b) are representations which illustrate the histology of Rhino mouse skins processed with H&E staining, (a) untreated, and (b) treated daily with 0.1% (w/v) trypsin in GDL liposomes for five days.

30           FIGS. 3(a) and 3(b) are color representations which illustrate the cross-sectional view of Rhino mouse skins which were processed for paraffin sections and stained for elastin. FIG. 3(a) is vehicle treated, and FIG. 3(b) is trypsin treated. FIGS. 3(c) and 3(d) are representations which illustrate the cross-sectional

5 view of C57Bl/6 mouse skins which were processed for  
paraffin sections and stained for elastin. FIG. 3(c) is  
vehicle treated, and FIG. 3(d) is trypsin treated.

FIGS. 4(a-c) are representations which illustrate  
the histology of Rhino mouse skins processed with H&E  
10 staining, (a) treated with 0.0005% (w/v) all-trans  
retinoic acid, (b) treated with 0.005% (w/v) trypsin,  
and (c) treated with both 0.0005% (w/v) all-trans  
retinoic acid and 0.005% (w/v) trypsin.

FIGS. 5(a) and 5(b) are representations which  
15 illustrate the cross-sectional view of the TUNEL-stained  
skin tissue of a vehicle treated Rhino mouse. FIGS.  
5(c) and 5(d) are representations which illustrate the  
cross-sectional view of the TUNEL-stained skin tissue of  
a Rhino mouse treated with trypsin.

20 FIG. 6 is a representation which illustrates the  
profile of gene expression of trypsin treated Rhino  
mouse skins at various concentrations of trypsin as  
detected by Reverse Transcription-Polymerase Chain  
Reaction ("RT-PCR").

#### 25 Detailed Description of the Invention

As used herein "(w/v)" shall mean grams of a given  
component per 100 ml of the total composition.

30 Topically active agents suitable for use in the  
compositions of the present invention as the first  
topically active agent include proteases, which include,  
but are not limited to, serine proteases. Preferably,  
the first topically active agent is selected from  
trypsin, carboxypeptidase-Y, protease IV, subtilysin, or

5 mixtures thereof. The protease of choice is trypsin.  
Preferably, the protease is present in an amount, based  
upon the total volume of the composition of the present  
invention, of from about 0% (w/v) to about 5% (w/v), and  
more preferably from about 0.01% (w/v) to about 1%  
10 (w/v).

While not wishing to be bound by any theory, it is  
believed that the first topically active agent of the  
present invention treats the hyperkeratinization  
associated with Acne Vulgaris and/or produces anti-aging  
15 effects on the skin. Though the first topically active  
agent can be used as the sole active ingredient in a  
composition for the treatment of Acne Vulgaris and/or to  
produce anti-aging effects on the skin, to more  
thoroughly treat Acne Vulgaris, the first topically  
20 active agent of the present invention can be combined  
with a second topically active agent.

Again, while not wishing to be bound by any theory,  
it is believed that said second topically active agent  
treats both the hyperkeratinization and the obstruction  
25 of the sebaceous follicle associated with Acne Vulgaris,  
while also producing anti-aging effects on the skin  
which are comparable to those produced by the first  
topically active agent. Thus, as evidenced by Example 6  
herein, the first feature of combining said first and  
30 second topically active agents is that the resulting  
treatment attacks at least two of the pathological  
processes associated with Acne Vulgaris, while not  
sacrificing the anti-aging benefits of the first  
topically active agent.

5           A second feature of combining said first and second  
topically active agents is evidenced by Example 4  
herein, which shows that combining said first topically  
active agent with said second topically active agent  
mitigates the irritant effect associated with said  
10 second topically active agent. Thus, the efficacy of  
treatment of Acne Vulgaris and/or signs of anti-aging  
effects on the skin are approximately the same with the  
treatments of the present invention as compared with  
treatments involving the second topically active agent  
15 alone, but the irritant effect normally associated with  
said second topically active agent is substantially  
reduced.

          A third feature of combining said first and second  
topically active agents is evidenced by Example 7  
20 herein, which shows that combining said first topically  
active agent with said second topically active agent  
substantially reduces the time necessary for product  
efficacy as compared to the use of the second topically  
active agent alone. Thus, the efficacy of treatment  
25 remains approximately the same as compared with  
treatments utilizing the second topically active agent  
alone, but the length of time required to see results  
normally associated with said second topically active  
agent is substantially reduced by combining said second  
30 topically active agent with said first topically active  
agent.

          Topically active agents suitable for use in the  
compositions of the present invention as the second  
topically active agent include those compounds in the

5 class of retinoids, which include, but are not limited  
to, retinoic acids, vitamin A alcohol, vitamin A  
aldehyde, retinyl acetate, retinyl palmitate, or other  
derivatives, analogs or mixtures thereof. The retinoid  
of choice is all-trans retinoic acid. Preferably, the  
10 retinoid is present in an amount, based upon the total  
volume of the composition of the present invention, of  
from about 0.0001% (w/v) to about 0.5% (w/v), and more  
preferably from about 0.001% (w/v) to about 0.025%  
(w/v).

15 If the delivery parameters of the first topically  
active agent so require, the pharmaceutical or cosmetic  
compositions of the present invention may preferably be  
further comprised of a pharmaceutically or cosmetically  
acceptable vehicle capable of functioning as a delivery  
20 system to enable the penetration of the topically active  
agent into the utriculus. While any commercially  
available vehicle for delivering the first topically  
active agent to the appropriate skin appendage, which in  
this case is the utriculus, is suitable for use as the  
25 pharmaceutically or cosmetically acceptable vehicle,  
liposomes are preferred. The liposomes are more  
preferably non-ionic and comprised of: (a) glycerol  
dilaurate or glycerol distearate; (b) compounds having  
the steroid backbone found in cholesterol; and (c)  
30 fatty acid ethers having from about 12 to about 18  
carbon atoms, wherein the constituent compounds of the  
liposomes are in a ratio of about 53:10:22 to about  
63:20:32, and preferably from about 55:12:24 to about  
61:18:30, respectively. Liposomes comprised of glycerol



5        dilaurate / cholesterol / polyoxyethylene-10-stearyl  
ether ("GDL") are most preferred. Preferably the  
liposomes are present in an amount, based upon the total  
volume of the composition, of from about 10 mg/mL to  
about 100 mg/mL, and more preferably from about 25 mg/mL  
10       to about 50 mg/mL. A ratio of about 58:15:27,  
respectively, is most preferred. Suitable liposomes  
may preferably be prepared in accordance with the  
protocol set forth in Example 2, though other methods  
commonly used in the art are also acceptable.

15        The above described liposomal composition may be  
prepared by combining the desired components in a suitable  
container and mixing them under ambient conditions in any  
conventional high shear mixing means well known in the art  
for non-ionic liposomes preparations, such as those  
20       disclosed in Niemiec et al., "Influence of Nonionic  
Liposomal Composition On Topical Delivery of Peptide  
Drugs Into Pilosebaceous Units: An In Vivo Study Using  
the Hamster Ear Model," 12 Pharm. Res. 1184-88 (1995)  
("Niemiec"), which is incorporated herein by reference  
25       in its entirety.

      In alternative embodiments, the pharmaceutical or  
cosmetic composition of the present invention may be  
optionally combined with other ingredients such as  
moisturizers, cosmetic adjuvants, anti-oxidants,  
30       surfactants, foaming agents, conditioners, humectants,  
fragrances, viscosifiers, buffering agents, sunscreens,  
colorants, preservatives, and the like in an amount  
which will not destroy the liposomal structure, if

5 present, in order to produce cosmetic or pharmaceutical products.

When used in combination with one another, the first and second topically active agents of the present invention can be applied to the skin of a mammal either simultaneously or at different times. For example, in a first instance, if daily treatment with the combination of the first and second topically active agents is desired, the first topically active agent can be administered in the morning and the second topically active agent can be administered in the afternoon. In a second instance, to serve as an example only, the second topically active agent can be administered in the morning and the first topically active agent can be administered in the afternoon. In a third instance, again, to serve as an example only, the first and second topically active agents can be administered together. In a fourth instance, serving only as an example, the first and second topically active agents can be administered on alternate days. Furthermore, in a fifth instance, serving only as an example, the treatments with the first and second topically active agents do not have to be given in a one-to-one dosage, so the first topically active agent can be administered for two days, while the second topically active agent is administered on the third day and so on. There are, of course, multiple variations of this fifth instance. The previous five examples are provided only to illustrate some of the many different treatment regimens possible with the methods of the present invention. It should be

5 understood that these examples are not limiting in any way to the treatment methods of the present invention, and that many other treatment regimens are possible.

10 The pharmaceutical or cosmetic composition should be applied in an amount effective to treat Acne Vulgaris and/or produce anti-aging effects on the skin. As used herein "amount effective" shall mean an amount sufficient to cover the region of skin surface where treatment of Acne Vulgaris and/or production of anti-aging effects is desired. Preferably, the composition is applied to the skin surface such that, based upon a 15 square cm of skin surface, from about 2  $\mu\text{l}/\text{cm}^2$  to about 8  $\mu\text{l}/\text{cm}^2$  of topically active agent is present when treatment of Acne Vulgaris and/or production of anti-aging effects on the skin is desired.

20 The invention illustratively disclosed herein suitably may be practiced in the absence of any component, ingredient, or step which is not specifically disclosed herein. Several examples are set forth below to further illustrate the nature of the invention and the manner of carrying it out. However, the invention should not be 25 considered as being limited to the details thereof.

#### Examples

##### EXAMPLE 1: The Rhino Mouse System

30 The Rhino mouse has been used as an experimental acne model to screen topically active comedolytic and antikeratinizing agents as described in Sundberg, J.P., "The Hairless and Rhino Mutations, Chromosome 14,"

5 Handbook of Mouse Mutations With Skin and Hair  
Abnormalities 291-312 (1994), which is incorporated  
herein by reference in its entirety. A recessive  
mutation on chromosome 14 results in a mouse with  
10 wrinkled skin devoid of body hair by age 25 days. At  
that time, the end of the first hair cycle, the  
follicular papillae fail to follow the regressing hair  
follicles and become isolated in the dermis. The  
papillae do not reassociate with the follicular  
epithelium to initiate a new hair follicle cycle. The  
15 upper remnants of the hair follicle are filled with  
sloughed, cornified cells and form utriculi with a small  
sebaceous gland at their base, resembling an open  
comedone. The rhino skin becomes progressively loose,  
forming folds and ridges, due to the expansion of the  
20 surface, secondary to abortive hair follicles filling  
with cornified debris. The utriculi progressively  
enlarge, forming pilary cists (pseudocomedones), which  
are dilated follicular infundibula filled with cornified  
debris.

25 RHJ/LE Hairless ("Rhino") male mice, 5-7 weeks of  
age, were obtained from Jackson Laboratories (Bar  
Harbor, Maine), and treated as described in Mezick et  
al., "Topical and Systemic Effects of Retinoids on Horn-  
Filled Utricle Size in the Rhino Mouse: A Model to  
30 Quantify "Antikeratinizing" Effects of Retinoids," 83 J.  
Invest. Dermatol. 110-113 (1984) ("Mezick"), which is  
incorporated herein by reference in its entirety.

EXAMPLE 2: Preparation of Topically Active Compositions

5           A sufficient amount of lyophilized trypsin,  
available from Sigma-Aldrich Corporation (St. Louis,  
Missouri), was mixed into a buffered aqueous solution of  
0.05 M N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic  
acid available from Life Technologies, Inc.

10       (Gaithersburg, Maryland) under the tradename "Hepes"  
such that the pH of the resulting solution was about 7.4  
and the concentration of trypsin in the solution was  
about 2% (w/v). One volume of the resulting trypsin  
solution was then mixed with one volume of (5%) glycerol  
15       dilaurate / cholesterol / polyoxyethylene-10-stearyl  
ether liposomes in water, which was prepared by the  
methods described in Niemiec, in order to yield a 1%  
(w/v) concentration of trypsin in the resulting  
topically active composition. The glycerol dilaurate  
20       was available from International Specialty Products Van  
Dyke (Belleville, New Jersey) under the tradename  
"Emulsynt GDL." The cholesterol was available from  
Croda, Inc. (Parsippany, New Jersey) under the tradename  
"Cholesterol VSP/NF." The polyoxyethylene-10-stearyl  
25       ether was available from ICI Surfactants Americas  
(Wilmington, Delaware) under the tradename "Brij 76."  
The volume to volume ratio of trypsin to GDL liposome,  
respectively, was altered to produce various  
concentrations of trypsin liposomal compositions.

30       Retinoic acid compositions contained an  
ethanol/propylene glycol vehicle which comprised 70%  
(w/v) ethanol (ethyl alcohol, 200 proof) which was  
obtained from Quantum Chemicals Corporation (Tuscola,  
Illinois) and 30% (w/v) propylene glycol which was

5 obtained from Fisher Scientific (Pittsburgh,  
Pennsylvania). The all-trans retinoic acid used in the  
retinoic acid compositions was obtained from BASF  
Aktiengesellschaft (Ludwigshafen, Germany). The volume  
to volume ratio of all-trans retinoic acid to  
10 ethanol/propylene glycol vehicle, respectively, was  
altered to produce various concentrations of retinoic  
acid compositions.

**EXAMPLE 3: Delivery of Trypsin into Hair Follicles**

15 About 100  $\mu$ L of the topically active trypsin  
composition of Example 2 was applied to the dorsal side  
of each Rhino mouse of Example 1. The trypsin used in  
this composition was fluorescently-labeled with a  
protein fluorescent labeling kit available from  
20 Molecular Probes, Inc. in accordance with its  
accompanying protocol (1996). At one and four hours  
after the application of the fluorescent trypsin  
treatment, a 1 cm by 2 cm sample of the skin surface of  
each mouse was isolated from each mouse with scissors,  
25 fixed with a 10% buffered formalin solution having a pH  
of about 6.9 - 7.1 at 25°C available from Stephens  
Scientific, then formed into a paraffin block according  
to well-known procedures, and examined with fluorescent  
microscopy according to well-known methods.

30 As shown in FIG. 1(a), almost all of the  
fluorescent labeling was found within the utriculi and  
sebaceous glands. The mice examined at the 1 hour  
interval (FIG. 1(a)) and the 4 hour interval (FIG. 1(b))

5 displayed identical histological staining patterns, with  
no additional skin penetration at the later time point.

This observation suggests against a possible non-  
specific extracellular matrix digestion by the protease,  
which would have likely shown a deeper penetration of  
10 the fluorescent stain into the stratum corneum at the  
later time point.

This Example was repeated on similar Rhino mice of  
Example 1, with the exception that these mice were  
treated daily for 5 days with the 1% (w/v) trypsin  
15 composition of Example 2 prior to the fluorescent  
trypsin treatment. Four hours after the application of  
the fluorescent trypsin treatment on the fifth day, the  
skins of these mice were analyzed using similar  
fluorescent microscopic methods. As illustrated in FIG.  
20 1(c), no major change was observed in the delivery route  
of the trypsin into the utriculi and sebaceous glands of  
the treated skins. However, the minimal staining at the  
outer portion of the stratum corneum of the trypsin-  
treated skins indicated some loss of barrier integrity.

25 This loss of barrier integrity is reflected in the  
values for transepidermal water loss ("TEWL") as  
described in Example 4 and Table 1 herein.

This Example shows that the application of a  
topically active composition containing trypsin to the  
30 skin surface of Rhino mice resulted in the delivery of  
the trypsin primarily to the utriculi and sebaceous  
glands, both after short term and long term use.

5     EXAMPLE 4: Trypsin Treatment Reduces the Size of  
      Utriculi           but Does Not Induce Dermal Irritation

      Rhino mice of Example 1 were topically treated with  
the trypsin compositions (0.001% (w/v) - 1% (w/v)) of  
10    Example 2 once daily for five days. Animals were  
sacrificed at day 8 and image analysis was used to  
quantify the reduction in utriculi size. For image  
analysis, whole mount epidermis was processed and  
microscopic measurements were taken according to the  
15    methods described in Mezick as well as Bernerd et al.,  
"The Rhino Mouse Model: The Effects of Topically Applied  
All-Trans Retinoic Acid and CD271 on the Fine Structure  
of the Epidermis and Urticuli Wall of Pseudocomedones,"  
283(2) Arch. Dermatol. Res. 100-107 (1991) and Bouclier  
20    et al., "Quantification of Epidermal Histological  
Changes Induced by Topical Retinoids and CD271 in the  
Rhino Mouse Model Using a Standardizing Image Analysis  
Technique," 4(2) Skin Pharmacol. 65-73 (1991) which are  
each incorporated herein by reference in their entirety.  
25    Empire Imagins Database version 1.1 was used on a  
Gateway 2000 P5-100 computer for capturing images.  
Image Pro Plus version 1.3 was used for measurements and  
Microsoft Excel version 5.0 for data processing. The  
mean utriculus diameter ( $\mu$ ) and the mean sebaceous gland  
30    size ( $\mu^2$ ) were calculated for each treatment group (3  
Rhino mice), using 5 random fields, two measurements per  
field, per animal. Percent reduction in utriculi  
diameter was calculated in accordance with the methods  
described in Finney, D.J., "Parallel Line Assays,



Statistical Method in Biological Assay," Charles Griffen & Company Ltd. 69-104 (1978) which is incorporated herein by reference in its entirety.

As shown in Table 1, trypsin induced a dose dependent reduction in utriculus size that reached a plateau at ~0.1% (w/v) trypsin. A further increase in trypsin concentration did not result in more than 55% reduction of utriculus size relative to liposomal control. A small reduction in utriculus diameter was observed in the liposome vehicle alone. A single trypsin (1% (w/v)) treatment had no effect on utriculus size reduction when analyzed seven days later (not shown).

**Table 1: Trypsin Induces a Dose Dependent Reduction in Utriculus Size**

Treatment	Utriculus Size Reduction (%) vs. Liposome Control	TEWL (g/m <sup>2</sup> h)
Trypsin 0.001% (w/v)	26.85 ± 4.38	29.53 ± 3.68
Trypsin 0.005% (w/v)	19.58 ± 3.06	26.40 ± 1.77
Trypsin 0.01% (w/v)	33.08 ± 2.15	28.53 ± 2.18
Trypsin 0.05% (w/v)	43.84 ± 0.62	36.57 ± 2.07
Trypsin 0.1% (w/v)	50.67 ± 0.83	42.80 ± 4.33
Trypsin 0.5% (w/v)	54.31 ± 1.33	36.23 ± 1.24
Trypsin 1.0% (w/v)	54.85 ± 1.02	42.00 ± 1.14
Liposome Vehicle	13.2 ± 1.82*	19.8 ± 1.14

\* Percent of utriculus size reduction of the liposome vehicle treatment was calculated relative to the untreated control

To further characterize the effect of trypsin on the Rhino mouse skin, we measured the transepidermal water loss ("TEWL") using an "Evaporimeter EPI" evaporimeter available from Servomed AB by first

5 normalizing the evaporimeter with the ambient humidity  
and then placing the probe on the dorsal skin of the  
test subject at which point a reading of TEWL was taken.

10 As shown in Table 1, TEWL increased in a dose  
dependent manner, with a plateau reached at ~0.05% (w/v)  
trypsin. This is approximately the same concentration  
for the maximal reduction in utriculi diameter. There  
was no correlation between TEWL increase and visual  
15 irritation. The minor scaling and erythema observed  
throughout these experiments were not dose dependent and  
remained low even at 1% (w/v) trypsin. Furthermore, the  
TEWL for trypsin treated mice was lower than that for  
retinoid treatment given alone.

20 Histological analysis of untreated, liposome  
control, and trypsin treated Rhino mice skins revealed  
major changes in the trypsin treated skins. H&E  
staining and histological analysis were performed using  
standard techniques as described in Sheehand and  
Hrapchak, 1980.

25 As shown in FIG. 2(b), the trypsin treated  
epidermis was hyperplastic with an increase in the  
number of cell layers of both the follicular epithelium  
and the epidermis when compared with the untreated  
epidermis shown in FIG. 2(a). Changes were observed  
30 mainly at the granular layer and the stratum corneum  
resulting in restored desquamation and improved skin  
structure. These epidermal changes are well-  
characterized markers for retinoid activity *in vivo*, and  
are associated with potential clinical efficacy. To

5 further support the assertion that trypsin is unrelated  
to dermal irritation, FIG.2(b) shows no inflammatory  
cells, which would normally be present in an irritation  
situation.

10 This example shows that trypsin causes a dose  
dependent reduction in the size of utriculi. A  
reduction in the size of the utriculi is associated with  
potential clinical efficacy of compositions for treating  
Acne Vulgaris. Therefore, this example further shows  
that trypsin is effective in the treatment of Acne  
15 Vulgaris. This example further shows that topical  
trypsin treatments do not induce skin irritation.

**EXAMPLE 5: Trypsin Treatment Results in Increased Skin  
Elasticity**

20 Rhino mice of Example 1 which were treated with the  
trypsin composition of Example 2 showed a noticeable  
effect in skin elasticity. To quantitate this effect, a  
cutometer analysis was performed. We used a cutometer  
available from Acaderm (Menlo Park, California), and  
25 employed the methods described in Couturaud et al.,  
"Skin Biomechanical Properties: *In Vivo* Evaluation of  
Influence of Age and Body Site by a Non-Invasive  
Method," 1 Skin Res. and Technol. 68-73 (1995) and  
Elsner et al., "Mechanical Properties of Human Forearm  
30 and Vulvar Skin," 122 Br. J. Dermatol. 607-614 (1990)  
which are both incorporated herein by reference in their  
entirety. Suction was applied through a 2 mm aperture  
and the corresponding skin displacement and recovery  
after release of the negative pressure were measured.

In human studies, an improvement in the ratios of deformation parameters  $U_a/U_f$  (skin fatigue, or total recovery from the load),  $U_r/U_f$  (biological elasticity, or elastic recovery after loading), and  $U_r/U_e$  (firmness, or improvement in the deformation resistance of the skin) indicates better tonicity and elasticity of the skin. The deformation parameters  $U_e$ ,  $U_f$ ,  $U_a$ , and  $U_r$  are dependent, in part, on skin thickness. Consequently, ratios were used for evaluation as described in Barel et al., "Suction Method for Measurement of Skin Mechanical Properties: The Cutometer," Handbook of Non-Invasive Methods and the Skin 335-340 (1995) which is incorporated herein by reference in its entirety.

As shown in Table 2, trypsin treatment resulted in an increase in all of these parameters, which reflects improved skin elasticity. While variations between animals were significant, the increase in cutometric properties was consistent, and increased with time and length of treatment.

**Table 2: Mechanical Properties of Trypsin Treated Rhino Skin**

Biophysical Parameter	Day 7		Day 12		Day 16	
	Untreated Control	Trypsin Treated	Untreated Control	Trypsin Treated	Untreated Control	Trypsin Treated
$U_a/U_f$	0.541±0.40	0.593±0.09	0.656±0.08	0.663±0.10	0.429±0.09	0.675±0.03
$U_r/U_e$	0.408±0.80	0.557±0.21	0.242±0.06	0.666±0.24	0.243±0.06	0.733±0.18
$U_r/U_f$	0.300±0.19	0.359±0.22	0.370±0.05	0.548±0.11	0.204±0.31	0.404±0.08

5

To further study this elasticity effect, skin sections of Rhino mice from Example 1 treated with the trypsin composition of Example 2 were stained for elastin on paraffin sections in accordance with the methods set forth in Kligman, L.H., "Luna's Technique, A Beautiful Stain for Elastin," 3(2) The Amer. J. of Dermatopathol. 199-200 (1981) which is incorporated herein by reference in its entirety.

10

15

As shown in FIG. 3(b), elastin fibers (stained purple) were increased in thickness and density around the utriculi and the sebaceous glands of the trypsin treated Rhino mice when compared to the untreated mice of FIG. 3(a).

20

This same experiment was performed with C57Bl/6 mice which were obtained from Charles River Laboratories (Kingston, New York) with similar results. FIGS. 3(c) and (d), the untreated and trypsin treated skins, respectively, show the results of elastin staining. Table 3 below shows the increase in skin mechanical parameters following the trypsin treatment.

25

**Table 3: Mechanical Properties of Trypsin Treated C57Bl/6 Skin**

Biophysical Parameter	Day 16	
	Untreated Control	Trypsin Treated
Ua/Uf	0.429±0.14	0.675±0.18
Ur/Ue	0.243±0.21	0.7335±0.02

Ur/Uf 0.204±0. 0.404±0.  
 26 1

This example shows that topical treatment with trypsin increases the elasticity of C57B1/6 and Rhino mouse skins. Skin elasticity is a property associated with anti-aging. Therefore, this example further shows that trypsin imparts anti-aging effects to the surface of the skin.

**EXAMPLE 6: Trypsin Acts with a Mechanism Different from that of Retinoic Acid**

The possible effect of trypsin on the sebaceous component of acne was evaluated using the hamster ear model system. Young golden Syrian hamsters, 45-55 grams upon arrival, were purchased from Charles River Laboratories (Wilmington, Massachusetts). The ventral side of the hamsters' right ears were treated daily with 10 µl of the trypsin composition of Example 2, five days a week for three weeks, while the left ears were used as untreated controls. As shown in Table 3, trypsin had no effect on the size of the sebaceous gland in this system.

**Table 3: Effect of Trypsin on Size of Hamster Ear Sebaceous Gland**

Treatment	Sebaceous Gland Size (µ²)	Percent Size Decrease (%)
Untreated	99112.4 ± 2904.0	N/A
Liposome Vehicle	94698.9 ± 4997.1	4.45 (vs. untreated)
Trypsin 0.5% (w/v)	95043.0 ± 4269.1	-0.36 (vs. liposome)

vehicle)

5 This example shows that trypsin had no effect on  
the size of the sebaceous glands in the hamster ear  
model system. It is well known that in this type of  
10 model, retinoids induce a dose dependent reduction in  
the size of the hamster ear sebaceous glands.  
Therefore, this example further suggests that trypsin  
functions with a mechanism different from that of  
retinoid compounds.

15 EXAMPLE 7: Trypsin and Retinoic Acid Exhibit an Additiv  
Therapeutic Effect

A first set of Rhino mice of Example 1 were treated  
with suboptimal doses of the trypsin composition of  
Example 2. As used herein, "suboptimal" is defined as  
20 levels of trypsin concentration below the optimum for  
utriculi size reduction as demonstrated in Example 4. A  
second set of Rhino mice of Example 1 were treated with  
suboptimal concentrations of the all-trans retinoic acid  
composition of Example 2. A third set of Rhino mice of  
25 Example 1 were treated with both suboptimal doses of the  
trypsin composition of Example 2 and the all-trans  
retinoic acid composition of Example 2. In this third  
set of Rhino mice, the trypsin and all-trans retinoic  
acid treatments were each administered daily, but at  
30 different times (i.e. trypsin in the morning and all-  
trans retinoic acid in the afternoon). Mice were  
sacrificed and their skins were examined histologically  
with the procedure set forth in Example 3.

5           As shown in FIG. 4(c), Rhino mice treated with both  
the trypsin and all-trans retinoic acid compositions  
showed much improved desquamation when compared to the  
trypsin and all-trans retinoic acid treatments given  
alone (FIGS. 4(a&b)), though the treatments given alone  
10 showed marked improvement over the untreated skin (FIG.  
2(a)). Furthermore, the histological analysis revealed  
far fewer open utriculi in the surface of treated skins  
than either treatment given alone.

15           This example shows that a combined treatment of  
trypsin and all-trans retinoic acid produces an additive  
effect on skin surface characteristics such as the  
number of open utriculi, which means that these  
compositions are effective in the treatment of Acne  
Vulgaris.

20           EXAMPLE 8: Trypsin Eliminates PCD in the Follicular  
Epithelium

          Rhino mice of Example 1 were treated daily with a  
0.1% (w/v) trypsin composition of Example 2 for five  
25 days and sacrificed at day eight.

          1 cm by 2 cm samples of the skins of untreated,  
vehicle treated, and trypsin treated mice were obtained  
via the procedure set forth in Example 3 then analyzed  
using a TdT-mediated dUTP-biotin nick end labeling  
30 ("TUNEL") stain procedure as disclosed in Gavrieli et  
al., "Identification of Programmed Cell Death in situ  
Via Specific Labeling of Nuclear DNA Fragmentation", 119  
Jl. Cell Biology 493-501 (1992) ("Gavrieli"). During  
this procedure, the prepared skin sections were stained



5 using an "ApopTag <sup>TM</sup> Plus *In Situ* Apoptosis Detection  
Kit" available from Oncor, Inc. as specified in the  
"ApopTag <sup>TM</sup> Plus *In Situ* Apoptosis Detection Kit"  
protocol by Oncor, Inc. (Feb. 1995), which is based upon  
the labeling of fragmented DNA ends as described in  
10 Gavrieli. FIGS. 5(a-d) show a histological analysis  
wherein the stain has a peroxidase end point (brown) and  
a methyl green counter-stain. The resulting  
representations of this are provided in FIGS. 5(a&b)  
which are vehicle treated and FIGS. 5(c&d) which are  
15 trypsin treated.

As illustrated in FIGS. 5(a-d), the TUNEL-stained  
samples defined apoptotic cells by both morphology  
(condensed or fragmented nuclei and cytoplasm or  
apoptotic bodies) and by the color of its stain  
20 (fragmented DNA within the condensed nuclei were stained  
brown). As shown in FIGS. 5(a&b), TUNEL staining  
revealed an unusually high level of apoptotic bodies in  
the follicular epithelium. Trypsin treatment resulted  
in the elimination of all the apoptotic bodies within  
25 the follicular epithelium and the restoration of  
programmed cell death ("PCD") at the granular layer  
(FIGS. 5(c&d)) as epidermal differentiation was  
restored.

This example suggests that trypsin could restore  
30 the balance between cell death and proliferation within  
the follicular epithelium and within the epidermis. One  
of the contributing pathological processes of Acne  
Vulgaris is hyperkeratinization, which may result from a  
shift in this balance. Therefore, this example further

5 shows that the ability of trypsin to restore the proper balance in epithelial cell death and proliferation may be a factor in its ability to treat Acne Vulgaris.

**EXAMPLE 9: Trypsin Induces Changes in Gene Expression**

10 Rhino mice of Example 1 were treated daily with trypsin compositions (0% (w/v), 0.0001% (w/v), 0.001% (w/v), and 0.01% (w/v)), as prepared in Example 2, for five days and sacrificed at day eight. The skins of vehicle treated mice and trypsin-treated mice were  
15 obtained as described in Example 3, then their total RNAs were extracted using "RNA Stat-60" reagent available from Tel-Test "B," Inc. as described in Chomczynski, "Single Step Method of RNA Isolation By Acid Guanidinium Thiocyanate-phenol-chloroform  
20 extraction," 162 Anal. Biochem. 156-59 (1987) which is incorporated herein by reference in its entirety. A sufficient amount of RNase-free DNase available from Promega, Corp. under the tradename "RQ1 RNase-free DNase" was then added to the extracted RNA from each  
25 mouse such that each respective product contained 200 ng of DNased-RNA using the procedure set forth in "RNase-free DNase" protocol published by Promega, Corp. (May, 1995). The resulting 200 ng of DNased-RNA was reverse transcribed ("RT") using the procedure set forth in  
30 "Superscript II Reverse Transcriptase" a protocol published by Gibco-BRL (now Life Technologies, Inc.) (April 1992), using random hexamers as random primers which are commercially available from Life Technologies, Inc.

The resulting RT products were then amplified via a polymerase chain reaction ("PCR") using about a 0.5 unit (per 100  $\mu$ l reaction) of a thermostable DNA polymerase which is commercially available from Perkin-Elmer-Cetus Corporation under the tradename "Taq polymerase," and about 0.1  $\mu$ mol/reaction of mouse glyceraldehyde-3-phosphate-dehydrogenase (G3PDH) primers available from Clontech Laboratories, Inc. ("Clontech"), or primers as set forth in Table 4 (using the conditions in Table 4 or in accordance with the procedures set forth in the protocol accompanying the primers from Clontech).

Table 5 illustrates some of the DNA primers used, the amount of  $MgCl_2$  required for the PCR reaction, and the length of the PCR cycle. Involucrin primers were as described in Marthinuss, et al., "Apoptosis in Pam212, an Epidermal Keratinocyte Cell Line: A Possible Role for bcl-2 in Epidermal Differentiation", 6 Cell Growth Diff. 239-250 (1995) which is incorporated herein by reference in its entirety.

**Table 4: DNA Primers Utilized in RT-PCR Assay**

DNA Primer (See attached Sequence Listing)	$MgCl_2$ (mM)	Cycle (min) @ $^{\circ}C$	Number of cycles	Seq. ID No.
Transglutaminase sense 5' AACCCCAAGT TCCTGAAG	2.5	1 @ 94; 2 @ 55; 3 @ 72	35	1
Transglutaminase antisense 5' TTTGTGCTGG GCCACTTC	2.5	1 @ 94; 2 @ 45; 3 @ 72	35	2
Elastin sense 5' TAAGGCAGCC AAATATGGTG	5	1 @ 94; 2 @ 45; 3 @ 72	35	3
Elastin antisense 5' ACCTGGATAA ATGGGAGAAA G	5	1 @ 94; 2 @ 55;	35	4

5           When necessary for better visualization, the  
resulting PCR products were precipitated with ethanol  
according to well-known procedures. When primers for  
G3PDH were used, only 10% of the PCR reaction products  
10       were used.

          The PCR products were then analyzed on 2%  
agarose/ethidium bromide gels according to methods well-  
known in the art in order to compare the level of  
expression of certain genes in skins of trypsin-treated  
15       and untreated mice. An RNA sample from the skin of a  
Rhino mouse that was not reverse-transcribed was used as  
a negative control for each PCR amplification. An RNA  
sample from the skin of a six month old Rhino mouse was  
used as a positive control when positive controls were  
20       not commercially available. The results of the gel  
analysis showed that the migration of the RT-PCR  
products on the gels was always identical to that of the  
positive controls, and to that of the reported amplicon  
sizes.

25           The relative quality of each respective RT-PCR  
reaction product was then compared by analyzing the mRNA  
level of G3PDH, a "housekeeping" gene, in each  
respective product. As illustrated in FIG. 6, G3PDH  
gene expression was found to be similar in all samples  
30       examined, which thereby enabled the analysis of the  
relative levels of gene expression for the desired  
genes.

5 Transglutaminase, an enzyme involved in the cross  
linking and formation of apoptotic bodies, displayed  
high mRNA levels in control animals, and was reduced to  
below detection level with increasing concentrations of  
trypsin. This shows that trypsin restored utriculi  
10 homeostasis and eliminated abnormally high levels of  
apoptosis in the follicular epithelium.

Elastin mRNA increased following treatment with  
increasing concentrations of trypsin. Therefore, new  
elastin is expressed following trypsin treatment which  
15 results in increased skin elasticity, as described in  
Example 5.

The level of involucrin, a marker of epidermal  
differentiation, was increased following trypsin  
treatment in a dose dependent manner. This indicates  
20 that normal epidermal turnover and differentiation were  
restored. Thus, trypsin restores the balance of  
epidermal differentiation as shown in Example 8.

This Example showed that the effect of trypsin on  
Acne Vulgaris and its anti-aging abilities may be  
25 understood by examination of the expression pattern of a  
series of genes over a range of trypsin concentrations.

Trypsin-induced changes in mRNA levels were clearly  
evidenced, indicating a regulatory role for trypsin in  
PCD, apoptosis, elastin expression, and epidermal  
30 differentiation.

EXAMPLE 10: Use of Compositions Containing Trypsin and  
All-Trans Retinoic Acid

5           Glycerol dilaurate/cholesterol/polyoxyethylene-10-  
stearyl ether liposomes are prepared in accordance with  
the procedures set forth in Niemiec, wherein the  
constituent compounds of the liposomes are in a ratio of  
about 58:15:27, respectively. Prior to mixing the lipid  
10 and water phases to form the liposomes of Niemiec, 0.1%  
(w/v) ascorbic acid is added to the water phase, and the  
ingredients listed in Table 5 are added to the lipid  
phase of the composition. The final pH of this  
composition is adjusted to a range of 4 to 7, and  
15 prefereably from 4.5 to 5.5 with a suitable buffer.

**Table 5: Ingredients Added to the Lipid Phase**

Ingredient	% (w/v)
Tretinoin	0.01
Methyl Paraben	0.10
Propyl Paraben	0.02
Butylated Hydroxytoluene	0.05

20           A second composition, which comprises 1.0g trypsin  
dissolved in a 0.05M Hepes buffer, at pH 7.4 (q.s. to  
100ml), is added to the liposome composition in a ratio  
of about 1 part of the second composition for every 8  
parts of the liposome composition. This final  
composition is suitable for immediate topical  
application.